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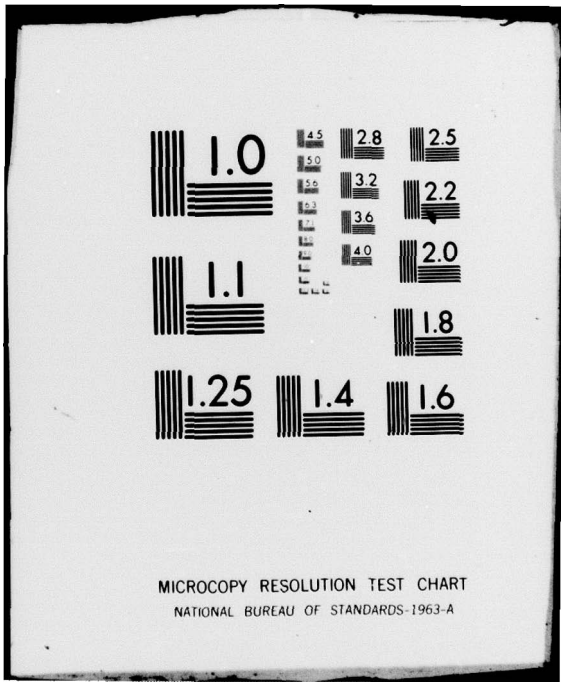
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The enzymatic degradation and structure of higher plant primary cell walls were the subjects of study. The enzymes employed included endopectate lyase, endo-β-1,4 galactanase, endo-β-1,4 xylanase and α-L-arabinofuranosidase. Cell wall material was from suspension cell cultures of Phaseolus vulgaris L. (bean) and Oryza sativa L. (rice). Extensive investigation of cell walls isolated from suspension cell cultures revealed that walls from both the dicot and monocot sources were quite heterogeneous with respect to cell wall types.

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and thus unacceptable for the proposed studies. Bean (dicot) cell walls proved to be much more susceptible to degradation by endopectate lyase than rice (monocot) cell walls. Other studies on the enzymolysis of these walls by other enzymes were not undertaken because they were considered to be too heterogeneous for the proposed studies. Preliminary investigation of other sources of primary cell wall revealed that tobacco pith cells represent a good source.

Procedures were developed for the purification of an α-L-arabinofuranosidase from Sclerotinia sclerotiorum. The endopectate lyase produced by Erwinia chrysanthemi was shown to be inducible and subject to catabolite repression. Also, the production of an exopolygalacturonase by this bacterium that attacks its substrate from the nonreducing end and releases digalacturonic acid was discovered; this enzyme was purified and characterized.

ENZYMOLOGY OF PLANT CELL WALL BREAKDOWN BY PLANT PATHOGENS

FINAL REPORT

D. F. BATEMAN and J. R. AIST

NOVEMBER, 1979

U. S. ARMY RESEARCH OFFICE

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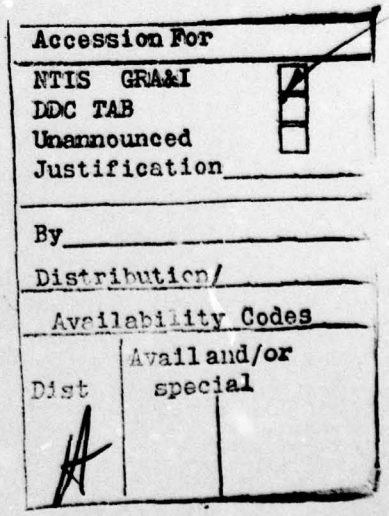

Forward

The broad objective of the work covered by this grant was to determine the role of specific polysaccharide degrading enzymes produced by plant pathogens in the degradation of the primary cell wall of higher plants. At the start of this program procedures had already been developed for the production and purification of the following enzymes: 1) endopectate lysae from Erwinia chrysanthemi (1), 2) endo-β-1,4 galactanase from Sclerotinia sclerotiorum (2), and endo-β-1,4 xylanase from Trichoderma pseudokaningii (3). Also, considerable progress had been made towards purifying an α-L-arabinofuranosidase produced by S. sclerotiorum. These enzymes - endopectate lyase, endo-β-1,4 galacterase, endo-β-1,4 xylanase, and α-L-arabinofuranoidase - represented the group believed necessary to break the bonds in the non-cellulosic structural polysaccharides important in the primary cell wall of higher plants.

Since cell walls produced in cell suspension cultures of higher plants in log phase growth had been reported to be an excellent source of primary higher plant cell wall (4, 5, 6), we proposed to prepare isolated primary cell walls from bean (Phaseolus vulgaris L.) and rice (Oryza sativa L.), a monocot and dicot, respectively, for use in studies to elucidate the role of specific polysaccharidases in primary cell wall breakdown. The plan was to use the above mentioned battery of enzymes individually and in different combinations and sequences, and follow the chemical and ultrastructural changes in primary cell wall as the enzyme(s) degraded wall structure. The results of these proposed studies were envisioned to help elucidate the role of the individual enzymes in wall degradation as well as contribute to a better understanding of higher plant cell wall structure.

To approach the primary objective of the program covered by this grant required the successful completion of several subobjectives: 1) preparation of the battery of needed enzymes, 2) preparation of isolated primary higher plant cell walls, and 3) determination of the chemical composition and ultrastructural characteristics of the cell wall preparations. At the present time the necessary battery of enzymes can be prepared. Our studies have shown that isolated cell walls from suspension cultured cells in log phase growth of both bean and rice are quite heterogeneous and thus unacceptable for the proposed studies. Preliminary work has indicated that walls prepared from tobacco pith cells are more uniform and may constitute an acceptable source of wall material for the studies as proposed in the original proposal.

The original objective of our proposal was not achieved, but significant progress was made along several lines which will contribute to designing a better approach to the problem. The work under this grant was interrupted about 2/3 through the grant period due to a change in employment of the Senior Principal Investigator.

Specific Contributions Made Under Grant Number DAAG 29-77-G-0219

1) Purification and characterization of an α-L-arabinofuranoidase

Studies revealed that Sclerotinia sclerotiorum produced α-L-arabinofuranoidase when grown at 25°C on a medium of mineral salts containing 0.4% L-arabinose plus 0.2% Difco yeast extract. This enzyme in 14-day-old

culture filtrates was concentrated and dialyzed by Amicon ultrafiltration. The specific activity of α-L-arabinofuranosidase was purified 26-fold by a four-step procedure: 1) preparative electrofocusing in granulated gel using Ampholytes with a pH of 7-9; 2) column ion exchange chromatography (Cm-Sephadex(C-50)) in 20 mM sodium acetate at pH 5.0; 3 and 4) two cycles of gel filtration on Ultragel (AcA54) in 72 mM phosphate buffer at pH 7.0 containing 100 mM NaCl. The purified enzyme had a pH optimum of 4.0-4.5, a molecular weight of 63,000 and a pI of 7.5. This purified enzyme released arabinose from both monocot and dicot cell walls, but did not macerate cucumber or potato tuber tissue. The details relating to the production, purification, and substrates for this α-L-arabinofuranoisidase have been published (7).

2) The heterogeneous nature of cell walls prepared from suspension cultured bean and rice cells

Cell suspension cultures of bean and rice were both grown in 40 ml of fresh Schauk-Hildelradt medium (8) in the dark at 26°C with shaking at 180 rpm. Cultures in log phase growth (5 and 12 days after transfer for rice and bean cultures, respectively) were used as a source for preparing cell walls. Cell walls were prepared according to the procedures of Talmadge et al (5). The composition of these cell walls is presented in Table 1. The results obtained agree closely with those published by others.

Isolated cell wall samples were prepared for electron microscopy by washing twice with 70 mM sodium phosphate buffer, pH 6.8, in nitrocellulose centrifuge tubes. Then 2.5% glutraldehyde in buffer was added and immediately subjected to centrifugation at 60,000 for 1 hr at 4°C. Following this, the pellets were subjected to four buffer rinses over a period of 1 hr and then fixed in 2% OsO_4 for 1 hr in 17.5 mM buffer. Pellets were washed with water for 30 min, dehydrated in an acetone series, and then diced into 1-2 mm^3 cubes. These cubes were further dehydrated, rinsed in propylene oxide, and embedded in Epon-Araldite-propylene oxide series. The plastic was polymerized at 70°C for 72 hr. Thin sections were prepared with a diamond knife, collected on 300 mesh Formar-coated copper grids, stained with uranyl acetate and lead citrate with or without pretreatment with barium permaganate (13). Sections were viewed in a Philips EM-200 electron microscope.

Both electron and light microscope studies of bean and rice cell wall preparations from cell suspension cultures revealed that both preparations were quite heterogeneous.

Light microscopy of bean walls showed the presence of some xylem elements with spiral, secondary thickenings as well as other types of walls with secondary wall depositions. Polarized light optics showed that most cell wall types were birefringent. At the electron microscope level, the thickness of cell walls varied considerably with the number of wall layers varying from 1 to 3, but occasionally as many as 6. Also, some walls stained more intensely than others. Wall laminae varied in texture and staining properties. Filaments, single or multiple, were associated with wall surfaces. Filaments were often seen unattached to the walls proper.

Examination of rice walls revealed similar heterogeneity. Rice walls usually retained their isodiametric shape and were often seen in clusters

Table 1. Composition of isolated cell walls from bean and rice suspension cultures

Component	Amount of total cell wall (% weight)[a]	
	Bean	Rice
Rhannose[b]	2.0	2.1
Fucose[b]	0.9	0.7
Arabinose[b]	17.5	22.3
Xylose[b]	3.9	16.5
Mannose[b]	0.6	0.3
Galactose[b]	5.4	7.4
Glucose (noncellulonic)[b]	5.7	7.2
Uronic acids[c]	12.1	6.6
Cellulose[d]	21.9	19.4
Protein[e]	18.8	12.2
Hydroxyproline[f]	2.5	0.1
Total	91.3	94.8

[a]Results calculated on a moisture corrected basis.

[b]Neutral sugars determined by the gas chromatographic method of Albersheim et al (9).

[c]Uronic acids were estimated with uronic acid dehydrogenase (10).

[d]Cellulose was determined using the method of Updegraft (11).

[e]Protein content was calculated by multiplying the Kjeldahl nitrogen content by 6.25.

[f]Hydrolyproline was analyzed by the method of Kivirikko and Liesmaa (12).

representing 3 to 8 cells. Wall layers usually varied from 1 to 3; some walls were observed with uniform secondary thickenings.

These detailed studies of bean and rice cell walls from cell suspension cultures demonstrated that such walls are quite heterogeneous and contain a great deal of wall material other than primary cell wall. These observations are contrary to what one finds in the literature (4, 5). We now feel that suspension cultured higher plant cells are not a suitable source of primary cell walls for the studies proposed in this program.

Preliminary studies were carried out to locate a more uniform source of higher plant cell walls for the proposed studies. Work with tobacco pith cells has revealed that they are highly uniform, both at the light and electron microscope levels. Also, treatment of isolated cell walls from tobacco pith with PL revealed that these walls are more or less uniformly susceptible to degradation by this enzyme. This is in contrast to the ununiform susceptibility of bean and rice cell walls from cell suspension cultures to this enzyme. We now believe that tobacco pith cells taken from a specified area of the tobacco stem (about 1/3 down the stem) can serve as a good source of higher plant cell walls for the studies originally proposed.

3) Susceptibility of cell walls of bean and rice from suspension cell cultures to endopectate lyase (PL)

While the above mentioned studies on bean and rice wall preparations were underway, studies on the susceptibility of these walls to degradation by PL were undertaken to determine the ultrastructural and biochemical effects of PL on both monocot and dicot primary cell wall. These studies were to be followed by others involving the various enzymes mentioned earlier individually and in different combinations.

A highly purified preparation of PL from E. chrysanthemi was prepared as previously described (1). One unit of PL released 1 µmole of uronide per minute when incubated at 30°C with 0.07% sodium polypectate in 33 mM Tris-HCl buffer at pH 8.5. Reaction mixtures contained 10 mg cell wall, 2 ml of 10 mM Tris-HCl buffer at pH 8.5, and 0.002 or 0.1 unit of PL. After designated periods of incubation, wall residues and supernatants were separated by centrifugation or vacuum filtration. The solubilization of wall carbohydrates was determined and wall residues examined by both light and electron microscopy.

Bean walls incubated with 0.002 units of PL lost 25% of their PL susceptible carbohydrate within 15 min, and nearly 50% after 60 min. This corresponded to a release of 13.5% and 27% of the noncellulosic wall carbohydrate, respectively. When incubated with 115×10^{-3} units of PL, rice walls lost 20% and 60% of their PL susceptible carbohydrate after 15 and 60 min, respectively. This corresponded to a 1.7% and 7.8% of the noncellulosic carbohydrate in the rice walls. Gas chromatographic analysis of reaction products revealed that galacturonic acid, rhamrose, arabinose, and galactose were solubilized from both wall preparations by PL (Table 2). Since this enzyme splits only the α-1,4 bond between galacturonic acid residues, the result confirms the expectation that the rhannogalacturonon chain contains side branches with arabinose and galactose constituents. The rice walls were much more resistant to PL than bean walls.

Table 2. Analysis of reaction products released from bean and rice cell walls by endopectate lyase after various periods of incubation

Incubation time	Bean			Rice		
	Unsat.[a] Uronide (µg)	Uronic[b] Acids (µg)	Neutral[c] Sugars (µg)	Unsat.[a] Uronide (µg)	Uronic[b] Acids (µg)	Neutral[c] Sugars (µg)
15	1.8	607	28	--	17	--
30		791	--	49.0	52	27
45	16.1	810	61	--	--	--
60	--	1095	90	60.71	103	44
90	28.7	--	130	--	--	--
120	35.5	--	158	77.2	218	66
Exhaustive	126.2[d]	--	360	118.6	--	103

[a]The amount of unsaturated uronide was estimated based on a molar extinction coefficient at 230 nm of 4,600 (1).

[b]Uronic acids were determined by the m-hydroxydiphenol assay (14).

[c]Neutral sugars were determined by gas chromatography of their alditol acetate derivatives (9).

[d]Reaction mixtures contained 100 mg of cell walls and 2.12 units of PL in 20 ml of 10 mM Tris-HCl buffer (pH 8.5). Incubation was for 24 hr in the presence of 0.02% sodium azide.

Histological analysis of bean walls following enzyme treatment revealed that 0.002 units of PL within 15 min caused many of the walls to swell and take on only a light stain. The laminated appearance of these walls was enhanced; wall fibrils became indistinct. Relatively few associated fibrils remained attached to wall surfaces; they disappeared. Some walls were degraded along their central core and some separated along the middle region. Other walls appeared uniformly degraded and appeared as faint ghosts. Yet other walls in the same reaction mixtures appeared unaffected by PL; this was especially true for those with secondary thickenings. Rice walls treated with PL, even at high PL concentrations, were less dramatically affected than the bean walls. Some rice walls exhibited some differentiation in staining in the middle lamella region. Walls treated with PL for 120 min appeared similar to those treated for 15 min, except that wall fibrils were less conspicuous. PL caused little to no swelling in rice walls.

In view of the heterogeneous nature of the wall materials and the differential reaction of walls in a given reaction mixture to PL, it was apparent that this system could not be effectively used to achieve the original objectives of the proposed studies. Thus, studies with other enzymes on these wall preparations were not undertaken. Preliminary studies with PL and isolated tobacco pith cell walls revealed that this enzyme solubilizes a major portion of the noncellulosic carbohydrates in these walls and that these walls react more uniformly to PL than did the bean and rice suspension-cultured cell walls. By the time this new source of wall material became available, the program was terminated because of a change in position of the Principal Investigator.

4) Satellite contributions resulting from Grant DAAG 29-77-G-0219

A) The pectic enzyme complex of Erwinia chrysanthemi and its regulation - E. chrysanthemi (strain 307) was the source organism for PL used in the above studies. Regulation of PL production was investigated. During logarithemic growth of strain 307 in mineral media on glycerol, poly-galacturonic acid (PGA), xylose, galacturonic acid, glycerol plus PGA, and glocose plus PGA, the respective differential rates of PL synthesis, as reflected by PL activity, were 0.00, 0.75, 0.00, 0.00, 0.76, and 0.02 μmoles min/mg bacterial protein. No PL was present in the sonicates of cultures growing on glycerol alone until the end of the logarithmic growth phase. Thus, PL synthesis was shown to be inducible and subject to catabolite regression. A factor in the medium of cultures growing on PGA, capable of diffusion through dialysis tubing, stimulated PL synthesis 2-fold in glycerol-sustained cultures simultaneously supplied PGA. Thus, PL induction appeared to be mediated by a PL reaction product. This conclusion was confounded by the observation that EDTA, which completely inhibits PL, had little effect on PL induction. These observations lead to the discovery of a low level of exo-polygalacturonase (exo PG) activity (c. 5% of PL) detectable by a reducing group assay performed in the presence of EDTA.

The exo PG has been purified, shown to have a molecular weight of 40,000 daltons, a pI of 8.5, and attacks PGA from the nonreducing end, liberating a dimer of galacturonic acid. Further work has shown that either saturated dimer liberated by endo PG or unsaturated dimer resulting from PL action can be taken up by the bacterium and cleaned lyitically releasing the

unsaturated monimer of D-galacturonic acid. The latter appears to be the inducer of PL. These studies will soon be published.

B) Improved procedure for measuring uronic acids in higher plant cell walls -

Current published procedures for measuring on a quantitative basis uronic acids in plant cell walls result in poor detection or they are non-specific and influenced by the presence of neutral sugars (14, 15, 16). We developed a procedure which utilizes uronate dehydrogenase (UAD) which specifically detects D-galacturonic and D-glucuronic acids. Since the oxidation of the above uronic acids is accompanied by a stoicheometric reduction of NAD, the procedure results in a sensitive assay for the uronic acids once they have been freed from the cell wall material in monomeric form.

Cell wall samples are first digested with a crude enzyme preparation that contains the necessary enzymes to hydrolyze cell wall carbohydrates to their monomeric constituents (17). This digest is then assayed for uronic acids with UAD. This procedure is more efficient than the gas chromatographic procedure (16), it avoids the hydrolysis of cell walls with strong acids, as used in m-hydroxydiphenol and carbozole assays (14, 15), which is often incomplete due to the unusual resistance of aldobuironic acids. The only disadvantage of the UAD assay for measuring uronic acids is that it fails to distinguish between D-galacturonic and D-glucuronic acids. Since the plant cell wall contains primarily D-galacturonic acid and only minor quantities of D-glucuronic acid, the UAD procedure coupled with the S. rolfaii digestion is the most efficient, easy to use assay devised to date for determining uronic acids in plant cell walls. This assay should have broad application in measuring uronic acids in biological samples. We plan to publish a detailed procedure for its application.

5) Publications resulting from Grant DAAG 29-77-G-0219

Baker, C. J., C. H. Whalen, R. Z. Kerman, and D. F. Bateman. 1979. α-L-Arabinofuranosidase from Sclerotinia sclerotiorum: purification, characterzation, and effects on plant cell walls and tissue. Phytopathology 69:789-793.

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Baker, C. J., J. R. Aist, and D. F. Bateman. 1980. Ultrastructural and biochemical effects of endopectate lyase on cell walls of bean and rice prepared from cell suspension cultures. Can. J. Bot. In Press.

Whalen, C. H., and D. F. Bateman. 1980. Determination of uronic acids in biological samples with uronic acid dehydrogenase. (Manuscript in preparation.)

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